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Tissue Distribution of Beta-Lactam Antibiotics

Experimental studies in rabbits

By Otto Cars

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TISSUE DISTRIBUTION OF BETA-LACTAM ANTIBIOTICS

Experimental studies in rabbits

AKADEMISK AVHANDLING

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av

Otto Cars
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ABSTRACT

Cars, O., 1981. Tissue distribution of beta-lactam antibiotics. Experimental studies in rabbits. *Scandinavian Journal of Infectious Diseases, Supplementum 27. Distributed by Almqvist & Wiksell Periodical Company, Stockholm, Sweden. ISSN 0300-8878.*

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The results also indicated that β-lactam antibiotics are not homogeneously distributed in muscle tissue but mainly confined to the extracellular fluid volume. Whole tissue levels could therefore underestimate the actual antibiotic concentrations in the tissue fluid where the bacteria causing soft tissue infections are most likely to be found. Serum levels, however, seemed to be a good indicator for the concentration time course in tissue fluid.

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This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Cars, O., Henning, C. & Holm, S.E.
Penetration of ampicillin and dicloxacillin into tissue cage fluid in rabbits: Relation to serum and tissue protein binding.
Scandinavian Journal of Infectious Diseases 13: 69, 1981.

II Cars, O. & Ögren, S.
A microtechnique for determination of antibiotics in muscle.
Journal of Antimicrobial Chemotherapy. (In press.)

III Ryan, D.M. & Cars, O.
Antibiotic assays in muscle: Are conventional tissue levels misleading as indicator of the antibacterial activity?
Scandinavian Journal of Infectious Diseases 12: 307, 1980.

IV Cars, O.
Tissue distribution of ampicillin: Assays in muscle tissue and subcutaneous tissue cage fluid from normal and nephrectomized rabbits.
Scandinavian Journal of Infectious Diseases. (In press.)

V Cars, O., Ögren, S. & Erlendsdóttir, H.
Assays of dicloxacillin in rabbit muscle.
(Submitted for publication.)

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INTRODUCTION

Since the discovery of penicillin by Fleming in 1929 (24) and the later isolation of the penicillin nucleus,6-amino-penicillanic acid, numerous semisynthetic antibiotics have been developed (57). Today, the family of β-lactam antibiotics comprises a large number of structurally related compounds among them penicillins, cephalosporins and cephamycins. In spite of the fact that these drugs have been widespread in clinical use for many years, e.g. benzyl penicillin for more than 30 years, the basis for their optimal dosage is still incomplete.

When a bacterial infection is treated with an antibiotic, the drug, the host and the microbes are involved in a complex system. Besides factors related to properties of the antibiotic, the outcome of an infection is e.g. influenced by a number of host factors, such as the nutritional state and the vitality of defence mechanisms and by microbial factors e.g. bacterial virulence, production of antibiotic-inactivating enzymes and other mechanisms for bacterial resistance.

Of major importance for the dosage are the toxicity, the antibacterial activity and the pharmacokinetic properties of an antibiotic, including tissue distribution. The toxicity of penicillins and other β-lactam antibiotics is low and does not normally limit the dosage. Serious toxic effects are only seen where high doses are given to patients with impaired renal function.

The antibacterial activity is usually expressed as the minimum inhibitory concentration (MIC). Antibiotic susceptibility tests are most often performed in vitro in static systems where a fixed inoculum of bacteria is exposed to a constant concentration of the antibiotic. The result of an MIC determination in the laboratory must therefore be interpreted with caution. In the infectious lesion the

the number of bacteria probably varies and the conditions of growth may be quite different from the in vitro situation. It is known, for instance, that changes in inoculum size, pH and oxygen tension influence the result of MIC determinations (4, 83).

The use of continuous monitoring of bacterial growth can give additional information about bacterial killing kinetics and may reveal effects of subinhibitory concentrations (74). Different in vitro models have also been used to study the response of bacteria to changing concentrations of antibiotics, such as occur in the body (3, 9, 30). Still, even with such models it is virtually impossible to simulate the normal physiological environment at the site of the infection. One way to circumvent these problems and the uncertain clinical importance of usual in vitro susceptibility tests is to predict the therapeutic activity from studies of experimental infections. Although this approach seems to be the most logical for evaluating the efficacy of antimicrobial agents, the choice of a good experimental model is difficult (10).

Some factors of importance for tissue distribution

Knowledge of the concentration time course of the antibiotic at the site of the infection is important for determining rational dosage schedules. A prerequisite for pharmacokinetic studies is that the drug concentrations can be followed for a sufficient time and, preferably, serial samples should be obtained from the same individual. Therefore, most such studies are based on repeated blood samples that are relatively easily accessible. The values of serum concentrations at different intervals after administration of the antibiotic can be fitted to pharmacokinetic models, which conceive the body as a compartmental system.

For most β-lactam antibiotics an open two-compartment model has been used, where it is assumed that exchange and elimination occurs from a central compartment surrounded by a peripheral "tissue" compartment (50). During elimination a pseudo-distribution equilibrium is assumed between the central compartment and the tissue compartment (28). Although studies on serum pharmacokinetics of antibiotics have contributed substantially to the knowledge of drug absorption, distribution and elimination they cannot predict the actual antibiotic concentration at specific body sites. Such detailed information can only be derived from direct determination of the antibiotic concentration in tissues.

Following an intravenous (i.v.) injection, the antibiotic will be distributed from the blood into extravascular sites. The transcapillary exchange of β-lactam antibiotics occurs by passive diffusion in most organs (5). The rate and extent of the distribution from blood to tissues will be determined by the concentration gradient and by properties of the drug: molecular size, protein binding, dissociation constant (expressed as pKa) and lipid solubility; and also by physiological factors such as capillary permeability, capillary surface area and rate of blood flow.

The role of protein binding of antibiotics to serum proteins has been a subject for discussion since the beginning of the antibiotic era. Although the influence of protein binding on drug tissue distribution and therapeutic efficacy remains controversial there seems to be a general agreement about several important properties of this binding. Hydrophobic binding to albumin appears to be primarily responsible (58) and the extent of binding of β-lactam antibiotics has been shown to correlate to the lipophilic character of the side chain (14). The fraction of an antibiotic bound to plasma proteins is considered to be biologically inactive and, also, only

the free (non-protein bound) fraction can pass readily through the capillaries to the extravascular spaces (39, 58, 60, 71). Thus, the levels of free drug in the tissues will be governed by the levels of free drug in serum, and at equilibrium, free concentrations will be equal on both sides of the capillary membrane (58). The total (free plus bound) levels in serum will be related to the extent of protein binding. Most of the albumin in the body is located extravascularly in the interstitial fluid (61). Thus, also in this fluid, protein binding will govern the total levels of drug achieved. In addition, binding to other constituents of the tissue may occur (19).

β-lactam antibiotics are acidic drugs, dissociated at physiological pH (35). The ionized form of the antibiotic molecule cannot pass the capillary endothelial cells but can penetrate the capillaries at most body sites through the capillary pores (32). At these sites pKa and lipid solubility may therefore be of secondary importance. Some areas of the body, however, appear to lack such pores, e.g. the brain, eye, intestine and prostate (5). In these areas, the transcapillary diffusion will be entirely dependent on the degree of ionization and lipid solubility.

Antibiotic assays in tissues

Determination of antibiotic concentrations in tissues have mostly been performed by microbiological methods. β-lactam antibiotics have been measured in various human body fluids such as pleural fluid, sputum, sinus secretions, wound fluid, synovial fluid etc. (36, 44, 46, 69, 75). Preparation of a valid standard is of the utmost importance for the accuracy of results obtained with microbiological assays (4, 37, 38). To measure the total amount of biologically active drug, the standard should have the same properties as the sample to be tested.

This may be relatively easy to achieve when the sample is a fluid, but is more difficult for assays of whole tissues.

For whole tissue assays, a variety of different techniques have been used. A few authors have applied the tissue specimens onto the agar surface (21, 34). However, most methods for determination of antibiotic content in tissues have included homogenization or extraction procedures of the tissue samples, which must consequently have an appreciable size. If only, for ethical and practical reasons, a small number of specimens can be obtained after administration of the drug, no evaluation of pharmacokinetics can be made. Nevertheless, tissue concentrations of antibiotics are often compared to the peak serum level or simultaneous serum level and expressed as a penetration ratio. This ratio may, however, vary depending on sampling time. Furthermore, binding of the antibiotic in serum and tissue must be taken into consideration which will be discussed later.

The limitations of whole tissue assays have encouraged the development of experimental models in order to provide data for a better understanding of the interaction between serum and tissue levels of antibiotics. Many bacterial infections start and progress in the interstitial tissue fluid, and this compartment has thus become a special focus of interest for studies of antibiotic tissue penetration. Various types of tissue cages implanted in experimental animals have frequently been used for such studies (16, 18, 68, 81). These models allow withdrawal of adequate and frequent samples at different times after administration of the antibiotic. Other techniques used for sampling of extravascular tissue fluid are skin windows (80), skin blisters (70, 72), implantation of paper discs (6, 64), and skin abrasions (29, 84). Recently, a method previously tested for assay of anti-

biotics in kidneys (49) was developed for measuring antibiotic concentrations in subcutaneous tissue fluids from animals and humans. With this model small volumes of tissue fluid are sampled by implanted cotton threads (65). The results of pharmacokinetic studies of β-lactam antibiotics in different tissue fluids are conflicting. For example, drug elimination from tissue cage fluid has been found to be much slower than from serum. In other models, however, kinetics in tissue fluid seem to be similar to that of serum (5, 29, 65), and the relevance of pharmacokinetic data obtained from experiments with tissue cages is a subject for discussion (23, 42, 64, 67, 84).

AIMS OF THE PRESENT INVESTIGATION

From the above review it is obvious that the experimental grounds for an optimal antibiotic dosing are inadequate. Despite its limitations, MIC is the parameter most frequently used as a basis for antibiotic therapy. Determinations of MIC are usually performed in protein-free media, and the non-protein bound fraction of the drug at the site of the infection should therefore be the one most relevant to compare with the MIC of the infecting organisms. Such data can, however, not be obtained from individual patients in clinical practice and dosage schedules for antibiotic therapy are instead usually derived from comparisons between measured serum concentrations and the bacterial susceptibility. Consequently, there is a need to study the relationship between serum concentrations of antibiotics and levels obtained in different tissues.

This investigation was carried out to study the pharmacokinetics of β-lactam antibiotics in rabbit tissues using different assay techniques in the same experimental animal. Two penicillins with known differences in serum protein binding (ampicillin and dicloxacillin) were chosen for the study in addition to a new, clini-

cally useful cephalosporin (cefuroxime). The study was limited to non-infected tissues.

The aims of the present investigation were:

(1) To study the penetration of ampicillin and dicloxacillin into tissue cage fluid in rabbits and the possible influence of serum and tissue protein binding on the pharmacokinetics (I).

(2) To develop a microtechnique for the determination of antibiotics in muscle tissue useful for pharmacokinetic studies (II).

(3) To investigate the relationship between antibiotic levels in muscle tissue and muscle tissue fluid (III-IV).

(4) To compare the pharmacokinetics of ampicillin, cefuroxime and dicloxacillin in rabbit serum and muscle tissue (III-V).

MATERIALS AND METHODS

Antibiotic assays

Antibiotics were obtained as sterile dry powders of known content. Ampicillin was supplied by Astra Läkemedel AB, Sweden, and Glaxo Research Laboratories, England; dicloxacillin by Bristol Laboratorier, Sweden, and cefuroxime by Glaxo Research Laboratories, England. All assays were performed with a microbiological agar diffusion method. Different test strains and culture media were used (I-V). Agar plates were prepared in 14 cm plastic petri dishes and a standardized inoculum of bacteria was either seeded into the agar before pouring the plates or flooded onto the dried agar surface. Test samples and standards were applicated into wells (2.5 mm diameter) in the agar. After incubation, the diameters

of the zones of inhibition were measured to the nearest 0.1 mm with vernier calipers. A standard curve was constructed by linear regression analysis (methods of least squares) of the inhibition zones of the different standard samples (Texas Instruments TI-51-III, electronic calculator). Concentrations in the test samples were then calculated from the standard curve.

Animals

All experiments in this investigation were performed with healthy albino rabbits of both sexes with a weight of 2.5 - 3.5 kg. Experiments involving sampling from muscle were performed under anaesthesia and the rabbits were sacrificed at the end of the experiments. A series of rabbits was functionally nephrectomized (IV) by bilateral ligation of the ureter and main renal vessels.

Blood samples were obtained from a marginal ear vein not used for injection of the antibiotic and collected in 0.1 ml non-heparinized glass capillaries. The capillaries were centrifuged within one hour and the serum separated. Aliquots (10 µl) of test samples and standards were pipetted into the agar wells. Standards were prepared by diluting the antibiotic stock solution with pooled rabbit serum (National Bacteriological Laboratory, Stockholm, Sweden).

Tissue cage fluid

Subcutaneous tissue fluid was obtained using a tissue cage model previously shown to give reproducible results and to be useful for pharmacokinetic studies (63). Under anaesthesia two 2 x 2 cm cylindrical steel net cages with an open surface area of 50 % (Fig. 1) were implanted in the subcutaneous space on each side parallel to the spinal axis. The rabbits were left untreated for the following 4-6 weeks, during which period the cages filled with fluid. In the course of the experiments, tissue cage fluid (TCF) was collected by percutaneous

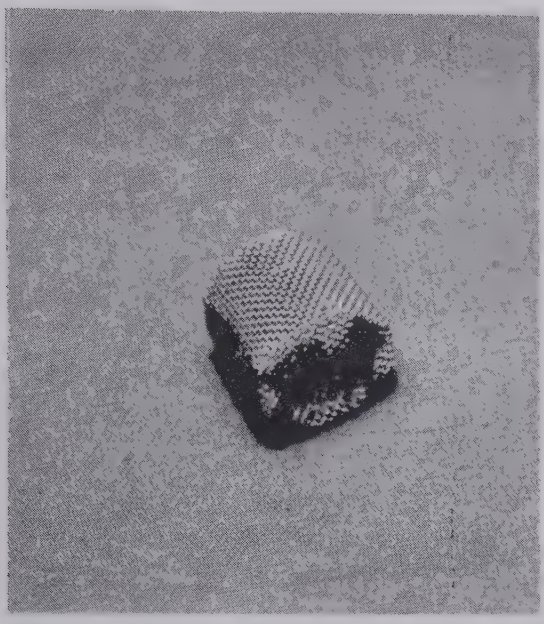

Figure 1: Cylindrical steel net cage used for subcutaneous implantation in rabbits.

aspiration with a syringe and fine needle. For antibiotic assays 0.02 - 0.03 ml fluid was usually removed from two cages at each time point. TCF samples were assayed as described for the serum samples. Standards were prepared in 50 % rabbit serum (rabbit serum diluted 1:1 with phosphate-buffered saline (PBS)) (I, IV).

Muscle tissue fluid

In the present study, a previously described method for antibiotic assays in subcutaneous tissue fluid by implantation of cotton threads (65) was applied for sampling of small amounts of muscle tissue fluid (III, IV). Pure cotton threads (0.2 mm diameter) were used. Before implantation, the animals were anaesthetized and the superficial muscle in one hind limb exposed by blunt dissection. Several threads of 3-4 cm length were carefully inserted close underneath the muscle fascia with a blunt needle. Care was taken to avoid trauma to the muscle tissue. Short pieces of thread were allowed to protrude

through the fascia and the muscle covered with a thin plastic film to avoid evaporation.

During the experiments one or two threads were removed at different times after administration of the antibiotic. The part of the thread being under the fascia was cut off and immediately transferred to small (2.5 ml) preweighed plastic tubes (Fig. 2) which were sealed and reweighed.

Figure 2: Cotton thread used for sampling of muscle tissue fluid. Threads were implanted under the muscle fascia and removed and transferred into preweighed plastic tubes at different times after antibiotic administration.

The uptake of muscle tissue fluid was calculated from the total weight of the thread and the known dry weight of thread. One cm pieces of thread were then inserted and coiled into the agar wells. For standards, pieces of thread, to which 2 µl of known different concentrations

of ampicillin diluted in 50 % rabbit serum had been added, were used and also placed into wells.

Muscle specimens

On anaesthetized rabbits the superficial muscle in one hind limb was exposed and divided by a longitudinal cut. Muscle tissue pieces were cut out by means of scissors and forceps (Fig. 3). Superficial blood was removed by directly patting the pieces gently on a filter paper or, if necessary, by cutting off the superficial layer of the tissue.

Figure 3: Tissue pieces cut out from the exposed rabbit muscle by means of scissors and forceps. Upper right: Small muscle pieces (10-20 mg). Bar indicates 1 cm.

For assay of small muscle pieces a direct agar diffusion assay was developed. Tissue pieces were placed into small (2.5 ml) preweighed plastic tubes which were sealed exactly one minute after removal of the sample. The tubes were then reweighed within one hour and the sample weight calculated. The size of the muscle pieces used for pharmacokinetic studies (III-V) were usually 10-20 mg (Fig. 3). Without previous homogenization, the pieces were placed into the agar wells, which were subsequently sealed with a drop of molten agar. Following incubation overnight, round and distinct zones of inhibition formed around the tissue pieces (Fig. 4). Several ways for preparation of standards were investigated (see experiments and results).

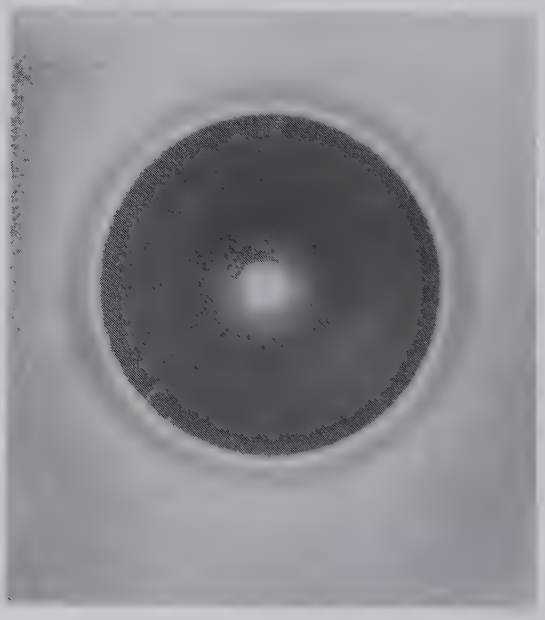

Figure 4: Zone of inhibition formed around an agar well containing a piece or rabbit muscle.

For comparison with the direct diffusion assay a conventional homogenizing technique was used. Muscle specimens (approx. 200 mg wet weight) were placed in sterile glass

tubes and 3 ml PBS per gram muscle wet weight was added. The specimen was homogenized using high-speed homogenizer (Ultra-Turrax^{R}), aliquots of the homogenate centrifuged for 10 minutes at 1000 x g and the supernatant decanted off. 10 µl aliquots of the supernatant were pipetted into the agar wells. Standards were prepared in muscle supernatant fluid obtained from homogenization of muscle tissue from rabbits not previously given antibiotics. These standard curves were found to be comparable to those obtained in PBS, and therefore the latter standards were used (II, V).

Determination of protein binding

Protein binding of ampicillin and dicloxacillin in rabbit serum and TCF (I) was determined by ultrafiltration with a 3 ml "stirred cell" (Millipore). Filters with a nominal molecular weight limit (NMWL) of 1000 were used and filtration performed at +37^{o} C with a positive pressure (nitrogen) under magnetic stirring. Antibiotic concentrations in the ultrafiltrates were determined as described above with standards diluted in PBS. The albumin concentrations of the ultrafiltrates were tested to exclude samples containing albumin as a result of improper filtration technique or defective filters.

EXPERIMENTS AND RESULTS

A microtechnique for determination of antibiotics in muscle

To investigate the evaporation of small muscle pieces (weights varying between 9 and 15 mg) the pieces were kept in the open air and their weights recorded from an electrobalance every half minute and plotted against time (II). The weight loss was found to be approximately linear with time and amounted to 1.74 $\pm$ 0.22 per cent per minute (mean $\pm$ S.D.) during the first five minutes after removal (Table I).

Table 1. Weight loss of muscle pieces due to evaporation (per cent per minute) during 1-5 minutes after recovery of the sample.

Sample No.	Time period			
	1-2 min.	2-3 min.	3-4 min.	4-5 min.
1	2.03	1.97	1.96	2.00
2	1.64	1.70	1.64	1.64
3	1.44	1.48	1.45	1.43
4	1.67	1.72	1.76	1.72
5	1.96	1.92	2.03	1.94
6	1.78	1.52	1.93	1.36
7	1.39	1.63	1.58	1.64
8	1.90	2.24	1.58	2.00
Mean	1.73	1.77	1.74	1.72
Total mean 1.74				
S.D. 0.22				

When the muscle pieces were placed into plastic tubes which were sealed, this prevented further evaporation and kept the weight constant for at least two hours (Table II).

Table II. Weights of sealed plastic tubes each containing one piece of muscle.

Item No.	Initial weight recorded at 0-15 minutes after removal of the sample (g)	Second weight recorded at 120-135 minutes after removal of the sample (g)
1	3.0769	3.0769
2	3.0381	3.0381
3	2.9935	2.9935
4	2.9575	2.9578
5	2.9931	2.9931
6	2.9931	2.9931
7	3.0620	3.0621
8	3.0118	3.0114
9	3.0816	3.0816
10	3.0703	3.0706

In the direct agar diffusion assay, the muscle pieces were placed into agar wells without previous homogenization. The reproducibility was improved when the wells were sealed with a drop of molten agar. Thereby it was ensured that the muscle had a firm contact with the agar and drying of the surface was prevented. Round and distinct zones of inhibition formed around the tissue pieces (Fig. 4).

Several ways for preparation of standards were tried (II). By adding known amounts of ampicillin to fresh muscle pieces or to pieces allowed to dry in room temperature for various periods up to 4 hours no reproducible standard curves could be obtained. To facilitate the re-

sorption of antibiotic into the tissue specimens, the pieces were first freeze-dried. During this process water evaporated whereby the pieces lost about 70 % of their wet weight. After freeze-drying, the volumes of ampicillin-containing solutions added to the muscle (5 µl) were always rapidly drawn into the dry tissue. Standard curves obtained by adding different amounts of ampicillin to freeze-dried pieces yielded highly reproducible results.

When these standard curves were compared to those obtained from dilutions in 50 % rabbit serum only slight differences were found which might well be accounted for by variance in the method (II). Therefore, for assay of ampicillin the less laborious standards in 50 % rabbit serum were used when assaying ampicillin in tissue pieces. A similar comparison for cefuroxime between standards prepared from freeze-dried tissue pieces and 50 % rabbit serum showed somewhat greater differences, the serum standards giving an average underestimation of the concentrations by 20 %. Since no equipment for freeze-drying was available when the pharmacokinetic studies with cefuroxime were performed (III) serum standards were used and all values subsequently adjusted. For assay of dicloxacillin standards prepared from freeze-dried muscle pieces were used (V).

For investigation of the reproducibility of the direct agar diffusion assay, rabbits were given antibiotic and sacrificed at different times after injection. Many small pieces were cut out from the same muscle and parallel assays performed. The coefficient of variation (S.D. as percentage of the mean concentration) for ampicillin assays with direct agar diffusion varied from 7.1 to 11.9 %. This variation was greater than for serum samples (coefficient of variation 3.8 %) but in the same range as that found for different homogenates (13.8 %)

(II). For dicloxacillin assays, the coefficient of variation for assay of muscle pieces was found to be 13.5 % (V). The results of direct diffusion assays of ampicillin and dicloxacillin agreed well with those obtained after homogenization (II, V).

Two methods were used to estimate the blood content of rabbit muscle. When rabbits were given ampicillin i.v. and samples from blood and muscle obtained for assay within one minute after injection (II), it was found that the total amount of ampicillin in muscle was only 0.9-1.7 % of the corresponding serum concentration, thus indicating a plasma mass of rabbit muscle of less than 1.7 %. In one rabbit, ^{131}I-labelled human albumin was injected intravenously and radioactivity measured in repeated samples from blood and muscle (V). The plasma volume of rabbit muscle thus calculated was 1.3 ± 0.8 % (mean ± S.D.).

Pharmacokinetics of ampicillin and dicloxacillin in subcutaneous tissue cage fluid

The penetration of ampicillin and dicloxacillin into TCF following i.v. injection was investigated at two dose levels, 10 and 40 mg/kg (I) (Figs. 5 & 6). Serum levels of both antibiotics declined rapidly. Ampicillin concentrations in TCF reached a maximum 30-60 minutes after injection and then declined with a half-life (T1/2) during elimination of 2.8 hours for the 10 mg/kg dose and 2.5 hours for the 40 mg/kg dose. With the fourfold larger dose, TCF levels of ampicillin were increased in the same magnitude, and the pharmacokinetics thus did not seem to be dose-dependent.

After injection of dicloxacillin, TCF concentrations increased more slowly and reached a plateau, which lasted for several hours before elimination occurred (Fig. 6). T1/2 during elimination was found to be as long as 25 hours in rabbits where TCF concentration was followed

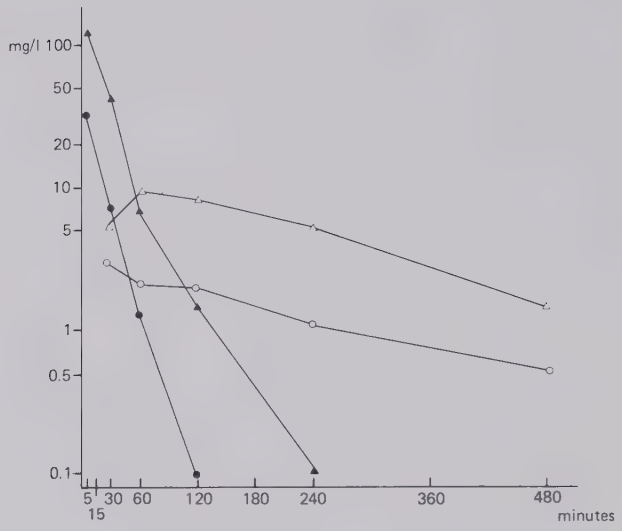

Figure 5: Serum concentrations (filled symbols) and TCF concentrations (open symbols) of ampicillin. Rabbits were given a single i.v. injection. 10 mg/kg: ●—○, 40 mg/kg: ▲—△

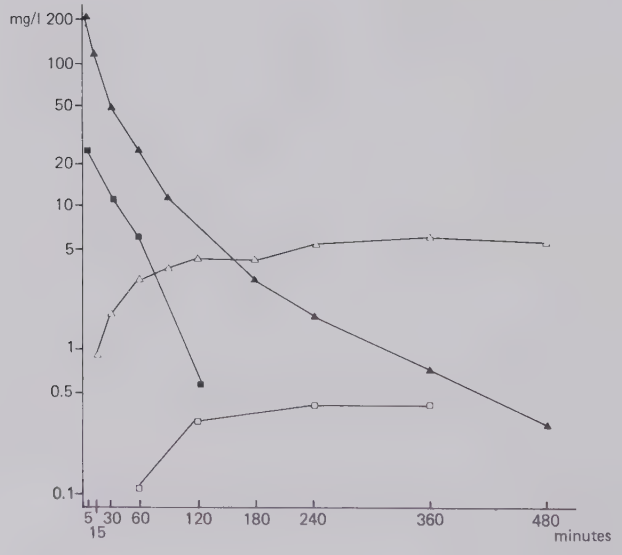

Figure 6: Serum concentrations (filled symbols) and TCF concentrations (open symbols) of dicloxacillin following a single i.v. injection. 10 mg/kg: ■—□ , 40 mg/kg: ▲—△

for a sufficient time for this calculation. In contrast to the results of ampicillin experiments, the pharmacokinetics of dicloxacillin in TCF seemed to be dose-dependent since an increase in dose of dicloxacillin from 10 to 40 mg/kg resulted in TCF concentrations more than three times above what could be predicted (I). This high outflow of the drug to TCF at the higher dose level could be explained by saturation of albumin binding at the early post-injection period, resulting in more free drug being available for transport.

The TCF/serum albumin ratio was found to be 0.57. Mean protein binding for dicloxacillin in serum was 98.6 % and in TCF 97.5 %. The corresponding values for ampicillin were 46.7 % and 45 %, respectively (I).

Ampicillin levels in muscle tissue and muscle tissue fluid

After intramuscular injection of ampicillin 25 mg/kg the concentrations were followed in serum, muscle pieces and muscle tissue fluid for 2.5 hours (III). Two important conclusions could be drawn from these experiments. Firstly, ampicillin was rapidly distributed into muscle tissue and muscle tissue fluid (Fig. 7). Secondly, the levels attained in the excised muscle pieces were many times lower than in muscle tissue fluid. The ratio between muscle concentrations and tissue fluid concentrations was calculated during elimination. The muscle concentrations were found to be 22 % of the corresponding levels in tissue fluid.

In a second series of experiments, ampicillin 20 mg/kg was given intravenously to rabbits (IV). Again a rapid penetration into the muscle was shown. Muscle levels were 16 % of concentrations in muscle tissue fluid. Elimination half-lives in serum and muscle tissue fluid were similar, indicating a rapid diffusion between the blood and the extravascular space.

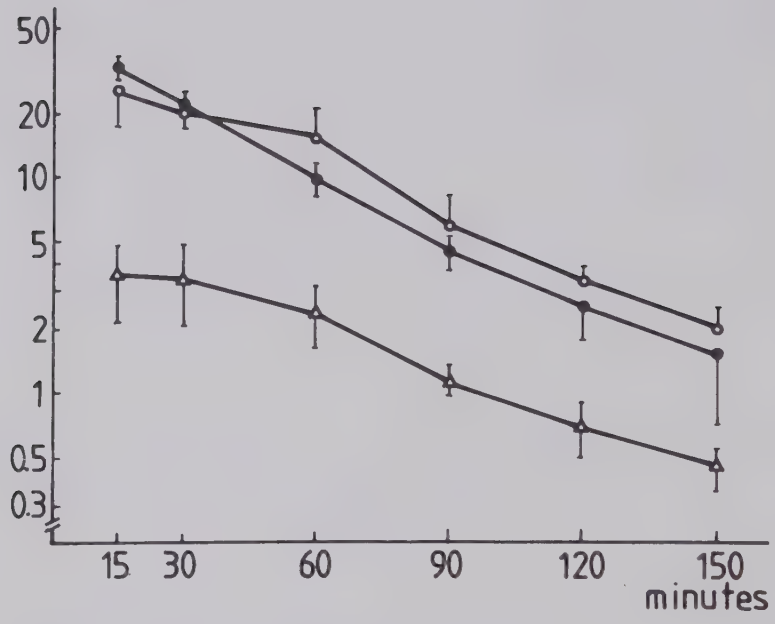

Figure 7: Mean concentrations of ampicillin following 25 mg/kg intramuscularly in 4 rabbits. Bars indicate S.D. Serum (µg/ml): ●—●; muscle tissue fluid (µg/ml): o—o; muscle (µg/g): △—△. (Scand J Inf Dis 12: 307, 1980).

In functionally nephrectomized rabbits (IV) given ampicillin i.v. there was an initial rapid decline in the serum levels and a corresponding increase in muscle levels. Ampicillin could be detected in muscle already after one minute and a steady state between serum and muscle seemed to be attained after 15-30 minutes. In contrast, the levels in TCF of functionally nephrectomized rabbits given ampicillin i.v. continued to increase slowly for several hours. Muscle tissue fluid levels in functionally nephrectomized rabbits were lower than in serum (Fig. 8). Assuming that a steady state was achieved, free levels should be at equilibrium, and the lower total concentrations in the tissue fluid may indicate a lower binding in this fluid. On this assumption, and using the previously found value of protein binding of ampicillin to rabbit serum (I), protein binding in muscle tissue

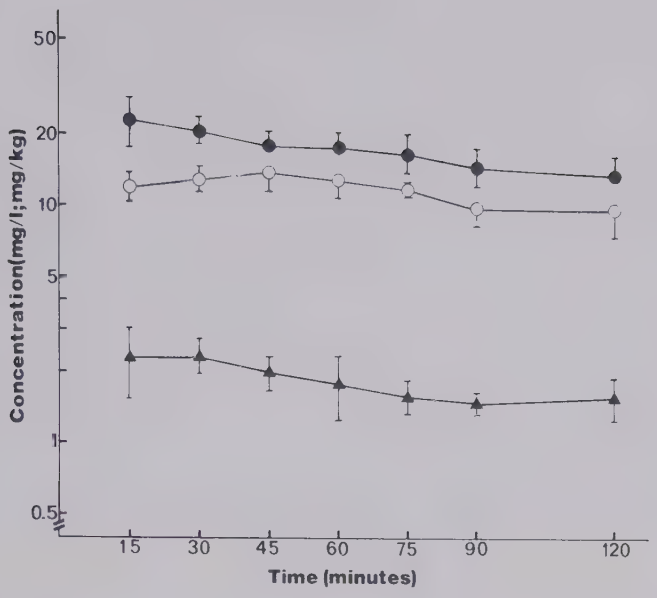

Figure 8: Ampicillin concentrations following 5 mg/kg i.v. in nephrectomized rabbits. Serum: ●—● (mg/l); muscle tissue fluid: o—o (mg/l); muscle pieces: ▲—▲ (mg/kg). Bars indicate S.D.

fluid was calculated and the value thus obtained was 25.7 %. Albumin concentration of rabbit muscle interstitial fluid is about 40 % of the serum level (13). This fact was used for calculating the protein binding in this fluid from formulas that do not require any assumption about distribution equilibrium (50). From these calculations, the same value (25.9 %) was obtained (IV).

Cefuroxime levels in muscle tissue and muscle tissue fluid

The distribution of cefuroxime into rabbit muscle tissue was studied following intramuscular administration of 25 mg/kg (III). A rapid distribution into muscle tissue similar to that found with ampicillin was also demonstrated for cefuroxime. Concentrations in muscle were lower than in muscle fluid. The levels in muscle were 18 % of corresponding tissue fluid levels.

Dicloxacillin levels in muscle tissue

Dicloxacillin was given as a single intravenous injection to groups of rabbits at three different dose levels, 10, 20 and 40 mg/kg (V). The increase in serum concentrations was proportional to the increase in dose. This was also true for the muscle levels, and thus no dose-dependent pharmacokinetics for dicloxacillin were found in muscle tissue. The penetration of dicloxacillin into muscle tissue was as rapid as for ampicillin and cefuroxime (III, IV) and half-lives in muscle and serum were similar. Preliminary results of our ongoing experiments have shown that, also for dicloxacillin, the levels in tissue fluid is several times higher than in whole muscle tissue (unpublished results).

DISCUSSION

Antibiotic assays in muscle

A microtechnique was developed for antibiotic assays in muscle tissue and the results compared with those obtained with subcutaneous tissue cages and implanted cotton threads. This tissue was chosen since it constitutes a large body compartment and is easily accessible. To obtain muscle samples from rabbits, the most practical technique was to expose the muscle and to cut out the samples. However, percutaneous sampling by the needle-biopsy technique described by Bergström (11) was also tried and found to be useful.

Certain precautions are required with small specimens in order to obtain accurate results. Evaporation decreases the mass of small specimens rapidly, and differences in evaporation time may thus seriously impair the reproducibility of tissue assays. This can easily be accounted for by eliminating the weight loss by sealing off the sample at a convenient time after harvest. It was not considered necessary to correct for the small weight loss introduced by evaporation during one minute (II).

Antibiotic assays in tissues are sometimes criticized because the blood contamination of the tissue has not been taken into account. The plasma volume in skeletal muscle obtained from sacrificed rabbits has been estimated to 0.4 % of the muscle volume (12). In the present study, estimation of blood content in skeletal muscle of living rabbits yielded only slightly higher values. Using ^{131}I-labelled albumin (V) the plasma volume of rabbit muscle was found to be 1.3 ± 0.8 % (mean ± S.D.) of the muscle volume, and this was not corrected for in the calculations. The low values obtained are consistent with the fact that, at a given moment, only a small fraction of the muscle capillaries are open for blood perfusion in resting skeletal muscle (31).

To measure the total amount of biologically active drug, the standard used in microbiological methods should have the same agar diffusion properties as the sample. For example, the protein concentration in the standard will influence the zone of inhibition obtained with highly protein-bound drugs (4, 37). For tissue assays, an ideal standard should contain a known amount of the antibiotic distributed exactly as the antibiotic in the tissue to be assayed, but this demand is difficult to fulfil. In the present investigation, a new principle for preparation of standards was tried. When rabbit muscle pieces were freeze-dried before addition of known amounts of antibiotic in volumes smaller than those lost during the lyophilization, highly reproducible standards were obtained (II, V). Delicate parts of the cells may, however, have been damaged during the freeze-drying process leading to an unphysiological distribution of the antibiotic in the standard specimen. However, it was shown that freeze-drying of muscle pieces did not hamper the diffusion from the tissue.

In short, the micromethod developed in this study has been found to be accurate and reproducible. Dilution of

the tissue samples is avoided which makes the assay sensitive. The results of the direct agar diffusion assay were comparable to those obtained after homogenization. The method, here applied to rabbit muscle has also been shown to be useful for pharmacokinetic studies in humans, because of the small amounts of tissue needed (43).

Relationship between serum and tissue fluid concentrations

Several techniques have been used to determine extravascular concentrations of antibiotics in tissues and body fluids. Particular interest has been directed towards studies on interstitial fluid concentrations. The extravascular space containing interstitial fluid has been described as a complex three-dimensional irregular lattice work of minute clefts interspersed between parenchymal cells and capillaries (56). Direct sampling of interstitial fluid is possible using micropuncture techniques (63). However, the amounts of fluid obtained are too small to permit assay of antibiotics with standard microbiological methods.

To be able to sample sufficient amounts of tissue fluid frequently, a variety of different methods have been developed such as tissue cages, skin windows and skin blisters (34, 55). Even if the tissue fluids that accumulate in the artificial spaces created with these models may be identical in composition to natural interstitial fluid, this does not necessarily infer that antibiotic levels obtained from such a model reflect the levels in a normal tissue. Ampicillin concentrations in tissue cage fluid obtained with the model used in the present study (I, IV) confirm the pattern of slow accumulation, lower peak TCF concentrations than in serum, and slow elimination found for ampicillin in similar animal models (17, 81).

It has been suggested that the characteristic profile of antibiotic levels in tissue cage fluid is due to a permeability barrier caused by a granulomatous tissue sheath surrounding the implanted cage (67). In the study of Alexander et al. (2) it was shown that antibiotic levels in implanted steel cylinders decreased with time after implantation. However, these differences were not considered to be caused by the development of a permeability barrier, but could be attributed to inconsistencies of the fluid volumes within the cylinders (2). Laber et al. used tissue chambers implanted only for a few days giving minimal cellular reaction in the surrounding tissue (41,42). Penetration of ampicillin into these chambers was also slow and peak chamber concentrations were low compared to the serum levels (42). Thus, it does not seem likely that a penetration barrier is the only explanation for the low antibiotic concentrations in tissue cage fluid.

Kinetics of ampicillin similar to that of tissue cage fluid with low and delayed peak concentrations have also been reported by authors using skin windows (78) and skin blisters (70). A possible explanation to the common pharmacokinetic features obtained with these different models is their relatively large volume, which could have a "diluting" effect on the antibiotic concentrations. The antibiotic molecules must reach the interstitial fluid through the capillaries. The capillary surface area available for drug diffusion in relation to the volume of tissue fluid will be much more unfavourable for the experimental models mentioned than in a normal tissue where the intercapillary distance is low. This could explain the relatively low concentrations of ampicillin and other β-lactam antibiotics achieved in tissue fluids with these models and in some tissue compartments of the body such as pleural and pericardial fluid (44, 66). The low diffusion area may also explain

the fact that drugs with a short serum half-life, like β-lactam antibiotics, exhibit a slower elimination rate from tissue cages than from serum (8).

In the present study cotton threads were used to sample small amounts of rabbit muscle tissue fluid for comparison with results obtained with tissue cages and muscle pieces. In contrast to TCF concentrations, the levels of ampicillin and cefuroxime found in the threads seemed to reflect a rapid dynamic equilibrium between serum and muscle tissue fluid. Results from experiments with cotton threads implanted subcutaneously (65) have shown that penetration of cefuroxime into subcutaneous tissue fluid is also very rapid and that the drug is eliminated at the same rate as from serum. Similar results have been obtained when subcutaneous tissue fluid has been sampled by filter paper discs (6, 64, 84). It seems likely that implanted threads and filter paper discs holding small fluid volumes represent more physiological techniques for pharmacokinetic studies of antibiotics in soft tissue fluids than e.g. tissue cages and skin blisters.

Relationship between whole tissue levels and extracellular fluid concentrations

The antibiotic content of tissue specimens is usually related to their wet weight and expressed as a concentration (e.g. mg/kg). This concentration should be equal to the concentration obtained in tissue fluid if the drug is evenly distributed within the tissue. However, it was evident from the studies of ampicillin and cefuroxime (III, IV) that the muscle fluid levels were several times higher than the levels obtained in whole muscle. The muscle levels of ampicillin were found to be 16-22 % of the concentration in tissue fluid and the corresponding value for cefuroxime was 18 %. This clearly shows that the distribution of these drugs in muscle

tissue is not homogeneous. This has also been suggested for cefazolin and cephradine (40).

If ampicillin and cefuroxime were exclusively located in the extracellular fluid, the volume of this fluid should be in the range of 20 % (16-22 %) of the muscle volume. Measurements of the interstitial fluid volume of rabbit skeletal muscle have, however, yielded somewhat lower values (7-14 %) (13, 62). This indicates that some antibiotic may also penetrate or bind to the muscular cellular mass. However, since the cell volume is large compared with the extracellular volume, effective concentrations will only be found in the extracellular fluid. In vitro studies of penetration of β-lactam antibiotics into tissue culture cells and leucocytes (15, 47) have also indicated that tissue cells are not well penetrated by these drugs. Additional support for the assumption that β-lactam antibiotics are distributed mainly within extracellular water is given by the fact that these drugs have low apparent volumes of distribution (5, 20). It has recently been stated, that β-lactam antibiotics do not distribute in extracellular water but in a larger volume, consistent with total body water (53). However, this was calculated on the assumption that no binding occurred in tissues, and if tissue binding (e.g. to albumin) is considered, lower values of the distribution volume would be obtained.

Antibiotic assays of serially obtained tissue specimens may give valuable information regarding the concentration time course. However, whole tissue levels of antibiotics give no information about the concentrations in the various compartments of the tissue. For ampicillin, cefuroxime and probably other β-lactam antibiotics whole tissue levels (e.g. obtained from homogenates or excised tissue pieces) will seriously underestimate the actual concentrations in the tissue fluid where the micro-

organisms causing soft tissue infections are most likely to be found.

The influence of protein binding on tissue distribution

Whether protein binding plays an important role for the distribution of drug from the blood to tissues is still a matter of dispute. There is an extensive literature on the binding of antibiotics to serum proteins but data on protein binding of antibiotics in tissues are scarce (1, 27, 36, 54). Although the protein concentration in tissue fluids is usually much lower than in the blood, this does not imply that protein binding in tissues can be neglected. In subcutaneous tissue cage fluid (albumin concentration 57 % of serum concentration) we found a significant protein binding of ampicillin and dicloxacillin (I).

At steady-state conditions, achieved e.g. by a continuous infusion of drug, free molecules will equilibrate over the capillary wall and free concentrations on both sides (i.e. in blood and in tissue fluid) will be equal unless special conditions prevail such as active transport (5) or a shift in pH (50). The total levels (free plus unbound fraction) of the drug in serum will be determined by the degree of protein binding.

In the tissue, the total levels of the drug at steady state will be dependent on the composition of the tissue fluid, and may thus differ from that of serum (58). For example, a lower protein content of the tissue fluid will result in lower total levels due to diminished protein binding. This was evident from the results of the experiments with functionally nephrectomized rabbits (IV). In these rabbits, devoid of the major elimination pathway of ampicillin, a steady state seemed to be attained (Fig. 8). The lower total levels in the tissue fluid was in accordance with the lower albumin content

in muscle tissue fluid (IV). This type of experiment (parallel assays in serum and tissue fluid at steady state) could be a useful tool for estimating in vivo protein binding of antibiotics in extravascular fluids. Our observations emphasize the importance to take protein binding into account when tissue concentrations of antibiotics are evaluated. A lower total level in a tissue fluid as compared to the serum level could, for instance, be misinterpreted to mean that the drug has a poor tissue penetration, while in fact the free levels in serum and tissues are equal.

A steady state between drug levels in serum and tissue is seldom achieved in clinical therapy with β-lactam antibiotics since intermittent dosage is the rule and these drugs are rapidly eliminated. In a dynamic situation, the importance of protein binding as a determinant of tissue distribution is more difficult to judge. Following a single dose of the antibiotic, free levels of drug may not be given sufficient time to equilibrate over the capillaries because of the rapid elimination of drug from the body. However, studies on cloxacillin, which is a highly bound β-lactam antibiotic, have shown that the free levels in lymph after a single dose are closely related to the free serum levels. This finding conflicts with the results of dicloxacillin assays in tissue cage fluid (I, 17).

In some studies where the tissue distribution of different β-lactam antibiotics have been compared in the same model, highly protein-bound drugs exhibit a slower penetration than drugs with a lower degree of binding (6, 16, 17, 36). This agrees with the results of the present investigation (I) which, in addition, showed a much slower elimination of dicloxacillin from TCF than for ampicillin. Related analogues with differences in protein binding may also vary with respect to other physico-chemical properties, such as lipid solubility, that can affect

tissue distribution (20). However, a substantial protein binding was shown for dicloxacillin in TCF (I) and the very slow elimination of this drug from TCF is likely to be due to a low diffusion gradient of free dicloxacillin. Support for this assumption is given by the fact that another highly protein-bound β-lactam antibiotic - cefazolin - was shown to be eliminated more slowly from extravascular fluid of high protein content than from fluid with low protein content (52). In addition, the dose-dependent pharmacokinetics found for dicloxacillin in TCF indicate that protein binding is of importance for drug diffusion in this model. In contrast to the findings in TCF, penetration and elimination of dicloxacillin in muscle tissue was rapid, and no dose-dependent pharmacokinetics were found (V). Also a rapid distribution and elimination has been demonstrated for cloxacillin and dicloxacillin in lymph and wound fluid (1, 48).

The restrictive effect of high protein binding upon tissue distribution and elimination of β-lactam antibiotics, suggested e.g. from studies with tissue cages, could be explained by the low area available for drug diffusion, which characterizes such models. Under these circumstances, variation in diffusion pressure (i.e. concentration of unbound drug) seems to have a measurable influence on the rate at which the concentration in tissue fluid is changed. In muscle tissue, however, no restrictive influence of high protein binding on the rate of tissue distribution could be shown (V).

Aspects on antibiotic dosing

Basic microbiological and pharmacokinetic data for an optimal dosage of antibiotics are still far from complete and many questions remain to be answered. The basis most frequently used for antibiotic dosing is that free serum levels should exceed the MIC for the infecting microorganism. However, to what extent the level in se-

rum should exceed the MIC and for how long is not well established (59). The advantages of continuous versus intermittent administration of antibiotics has been discussed for many years. A possible important factor for antibiotic dosing is the recovery period following exposure to antibiotics. This post-antibiotic suppression of bacterial growth, which may last for a few hours, has been known since many years (22) and has recently been reinvestigated (26). The studies of Weinstein et al. (82), which showed persistence of penicillin activity in fibrin clots for longer periods of time than in blood, also supported an intermittent administration of β-lactam antibiotics, which is today the most common one. As previously discussed, similar pharmacokinetics have been found for β-lactam antibiotics in other experimental models.

The present investigation was focused on the pharmacokinetic aspects of antibiotic dosing, particularly the relation between serum and tissue levels of β-lactam antibiotics. The pharmacokinetics of ampicillin, cefuroxime and dicloxacillin in muscle tissue was similar to that of serum. A rapid disappearance from muscle has also been shown for benzyl penicillin by Ullberg (76) who studied the distribution of this drug in different tissues using autoradiography.

Concentrations of β-lactam antibiotics in the extracellular fluid of soft tissues (muscle and subcutaneous tissue) seem to be well reflected by the serum levels, when methods that minimize disturbance of the physiological situation (i.e. cotton threads and paper discs) are used. It should be pointed out that the experiments in the present investigation as well as those hitherto discussed, were performed in normal tissues. Infection and inflammation may alter factors of importance for the antibiotic levels achieved in the tissue. In infected tissues, the possibility of antibiotic inactivation by

tissue constituents (45) or by bacterial enzymes (68) must be considered. It has also been shown, that infection decreases antibiotic concentrations in tissue cages (34, 51). Lundberg and Malmborg (46) showed lower concentrations of antibiotics in purulent sinus secretions than in mucous secretions. This was probably due to reduced blood circulation in the mucous membranes, due to oedema in the mucosa and strangulation of the vessels passing the ostium to the sinus (46). In a study, where artificial tissue oedema was produced (77) penicillin levels in aspirated tissue fluid could be detected much longer than in the blood. Similar penicillin kinetics in wounds were found by Florey et al. (25) when the drug was injected after severe tissue damage.

A number of factors may evidently influence the antibiotic concentrations achieved in a diseased tissue, and the clinical relevance of pharmacokinetic studies of antibiotics in tissue fluid obtained with different models is not firmly established. The concentration time course of β-lactam antibiotics in experimental abscesses resembles that found in tissue cage models (6, 76). The kinetics found in non-infected tissues in the present study (III-V) and previous reports (64, 65) seem, however, to agree with the pharmacokinetics of β-lactam antibiotics in infected muscle (40) and infected subcutaneous tissue (43). Also in infected wound fluid antibiotic levels similar to those in serum have been reported (69). For antibiotic dosing, serum levels of β-lactam antibiotics thus seem to be a good indicator of the concentration time course of these drugs in infected soft tissues, and the most relevant parameter for comparison with MIC seems to be the free serum levels. To confirm this assumption, there is a great need for additional kinetic studies of antibiotics in infected tissues.

SUMMARY AND CONCLUSIONS

Tissue distribution of ampicillin, dicloxacillin and cefuroxime was studied in rabbits. Three different methods for determination of antibiotics in tissues were used. Subcutaneous tissue fluid was obtained from implanted steel net tissue cages. Muscle tissue fluid was sampled by implantation of cotton threads under the muscle fascia. In addition, antibiotic concentrations were determined in small muscle tissue specimens. The antibiotic concentrations in tissue fluids and muscle tissue obtained at intervals after a single intravenous or intramuscular injection were compared to the concentration time course in serum. The results obtained and the conclusions drawn can be briefly summarized as follows:

1. A microtechnique was developed for antibiotic assays in small muscle tissue specimens. Serial samples (10-20 mg) of muscle could easily be obtained from the same experimental animal. Without previous homogenization, the samples were placed into agar wells in plates inoculated with the test strain. The results obtained with this direct agar diffusion assay correlated well with those of a conventional homogenizing technique.

2. Levels of ampicillin and dicloxacillin in subcutaneous tissue cage fluid were low as compared to the serum levels. Also, maximum concentrations occurred later than in serum and elimination was slower. These differences were more pronounced with the more highly protein bound drug, dicloxacillin. The low capillary surface area available for drug diffusion in relation to fluid volume, which characterizes tissue cages, could explain the pharmacokinetic features obtained with this model.

3. In contrast to the results obtained from experiments with tissue cages, assays of ampicillin and cefuroxime

in muscle tissue fluid showed a rapid distribution of these drugs into the extravascular fluid and an elimination rate similar to that of serum.

4. A rapid distribution into muscle tissue was demonstrated for all three investigated β-lactam antibiotics. However, for ampicillin and cefuroxime it was shown that the concentrations in muscle tissue were several times lower than the corresponding levels in muscle tissue fluid. This indicated that these drugs, and possibly other β-lactam antibiotics, are not homogeneously distributed in the tissue, but mainly confined to the extracellular fluid volume.

5. Whole tissue levels could therefore underestimate the actual antibiotic concentrations in the tissue fluid, where the bacteria causing soft tissue infections are most likely to be found. Serum levels, however, seemed to be a good indicator for the antibacterial activity of the tissue. Further investigations with infected tissues are needed to confirm this assumption.

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